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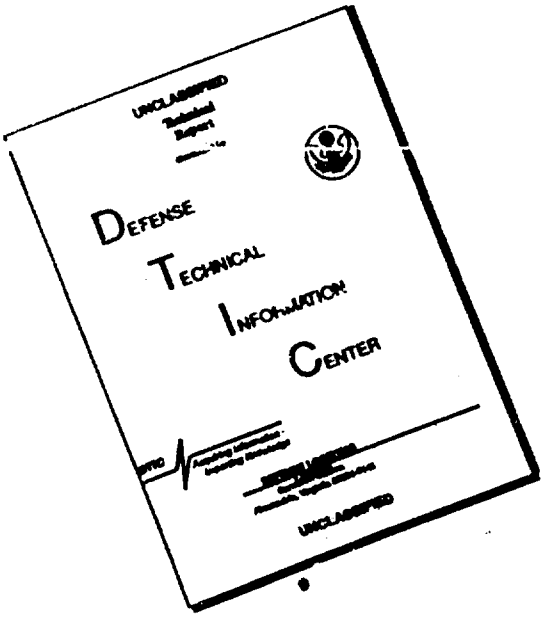

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THE NATURE OF ULTRAVIOLET LUMINESCENCE OF CELLS

[Following is the translation of an article by S. V. Konev, T. I. Lyskova, and V. P. Bobrovich, Laboratory of Biophysics and Isotopes, AN BSSR, Minsk, published in the Russian-language periodical Biofizika (Biophysics), Vol VIII, No 4, 1963, pages 433-440. It was submitted on 3 Dec 1962.]

In the works by Ye. M. Brumberg et al. ultraviolet luminescence of living plant, animal, and microbial cells was detected by the method of ultraviolet microscopy. The luminescence was excited by short-wave ultraviolet waves with wavelengths of 250-280 nm [1-11].

Almost simultaneously with the works of Brumberg et al. ultraviolet luminescence of suspensions of yeast and microbial cells was noted by Vladimirov using the spectrofluorometric method [12]. Subsequently research on the ultraviolet luminescence of biosubstrates, conducted mainly by the school of Ye. M. Brumberg, was carried out along two main paths: establishment of possible correlations between the luminescence picture and the functional state of the cell and the organism as a whole, on the one hand, and interpretation of the elementary nature of luminescence centers on the other.

In spite of the fact that in work along the first direction the study was mainly only of one parameter of luminescence - its intensity - by the present time a certain amount of factual material had been accumulated which made it possible to consider that cell luminescence included an expression of a change in their functional state during the processes of active life. For example, there were records of regular changes in the intensity of cell luminescence during the process of embryogenesis [13, 14], and changes were noted in the intensity of luminescence of the nucleus and the cytoplasm in the case of malignant growth [3, 4, 15], after irradiation with ultraviolet rays and ionizing radiation [3,7, 16, 17]. All of this strengthens still more the interest in the study of the nature of the centers which are responsible for the ultraviolet fluorescence of a cell.

Brumberg and coworkers proposed that the fluorescence of cell nuclei is conditioned by nucleic acids, and that of the cytoplasm by RNA and proteins [4]. However, subsequent experiments, demonstrating that the gradual removal of free nucleotides, RNA, and DNA from the cell is accompanied by a sharp drop in the intensity of luminescence, led to the conclusion that the fluorescence of protein in the cell is weak and cannot compete with the fluorescence of free nucleotides and RNA. This conclusion was soon contradicted by the fact that in the spectra of excitation of fluorescence of

cells of rat bone marrow, hepatic cells and spermatids of mice [8] maximum is displayed at 280-290 nm. These experiments should have reestablished to a known degree the exclusive right of protein in the mechanisms of development of cell luminescence, if it was not known that in a number of cases nucleic acids and nucleoproteins [18-20] may have a maximum in the spectrum of excitation in the same range (280-300 nm). Finally quite recently still another proposal emerged: the responsibility for luminescence of cells lies on the oxidized forms of pyridine nucleotides [21, 22].

Thus by the present time several points of view have been formulated on the nature of substances which are responsible for the luminescence of cells and their elements. These substances were found to be: in nuclei - a luminescent form of DNA [3, 5], free nucleotides [3], and proteins [8, 15]; and in the mitochondria and cytoplasm - free nucleotides [4], proteins [8], and an oxidized form of pyridine nucleotides [21, 22].

For the purpose of increasing the clarity in the interpretation of the nature of the ultraviolet luminescence of cells we made an attempt to investigate the luminescence of suspensions of isolated mitochondria and cell nuclei, using several spectral characteristics simultaneously; spectra of luminescence, spectra of excitation of fluorescence, and polarization spectra of fluorescence (based on absorption). A comparison of the stated spectral characteristics, reflecting the average picture of luminescence based on many thousands of similar cellular elements, makes it possible to come close to a single conclusion on the nature of the elementary emitters of the cell.

Objective and Method of Operation

In the work we investigated the mitochondria and nuclei of liver from adult white rats which had not received food for 24 hours prior to the experiment. For removal of erythrocytes the liver was carefully flushed with physiological solution through the portal vein. The hepatic tissue was ground in a homogenizer on a ground-glass joint. Cellular elements were separated by differential centrifugation. Cell fragments were precipitated from an 0.25 M solution of saccharose with 0.005 M of EDTA at 400 g, nuclei at 800 g for 10 min, and mitochondria at 8500 g for 20 min. The resulting fractions of nuclei and mitochondria were subjected to a two-stage repeated grinding and centrifugation for increasing the degree of purity. All the preparation work was carried out in a cold room at 4°. Identification of mitochondria and nuclei and evaluation of the degree of purity of the preparation were performed microscopically on unfixed smears and smears, fixed and dyed with azure-eosine, fuchsin and based on the Feulgen reaction. The smears were taken from the appropriate fractions. For the mitochondria complete purity was achieved from admixtures of fragments from cells, nuclei, and microsomes. Present in the fraction of nuclei was an insignificant amount of cell fragments (< 10%).

Spectral-luminescent measurements were made in suspensions of mitochondria and nuclei in a physiological solution or in 0.25 M saccharose. Spectra of luminescence were taken from the forward wall of a cuvette in a device which was described earlier [23, 24]. Luminescence was excited by a spectral band of 250-280 nm, separated from the integral flux of a SVD-120A mercury-quartz lamp with the help of a gaseous chlorobromide filter and a liquid Bekshtrem filter, and recorded with a liquid nitrogen cooled FEU-18A photomultiplier working under the conditions of a photon counter. Pulses, amplified by a wide-band "Siren" amplifier, were received on a "Tyulpan" pulse integrator. Recording of spectra was done with an EPP-09 recording potentiometer.

Low-temperature spectra of luminescence were taken in the following manner. The preparation, frozen in a metallic holder, was placed in a quartz Dewar vessel which was filled with liquid nitrogen. The exciting light which passed through the gaseous and liquid light filters was focused by a quartz short-focus lens through an aperture of optical quartz onto the frozen surface of the preparation. The light of luminescence was focused from that same surface of the preparation onto the inlet slot of an SF-4 spectrophotometer by an aluminezed parabolic mirror. After the inlet slot of the spectrophotometer the light of luminescence, after additional focusing, fell on the photocathode of the photomultiplier. In the tests for studying the dependence of spectra of phosphorescence on the wavelength of exciting light the monochromatic beams of exciting light were separated with the help of an SF-4 monochromator and the luminescence was dissociated into spectra with the help of an UM-2 monochromator. The spectral band width in the case of nonmonochromatic excitation did not exceed 0.3 nm; when working with two monochromators the spectral band width for exciting light comprised 1-3 nm, and for light of phosphorescence 1-2 nm.

The spectra of excitation of the integral flux of fluorescence (320-400 nm) were taken in transmitted light on a device which was described earlier on very diluted suspensions of nuclei and mitochondria (at 280 nm no more than 10% of light was absorbed). The spectral band width for exciting light varied within the limits of 0.1-0.3 nm.

Spectra of polarization of fluorescence were taken on a device which was described earlier [23]. Recordings were made of polarization of fluorescence which was transmitted through an interference light filter at 355 nm with a halfwidth of passage of 12 nm. The spectral band width for exciting light for the range of 254-302 nm comprised 0.2-0.3 nm, and for the range of 235-248 nm - 0.4-0.7 nm. Figures 1-3 show the spectra without corrections for spectral sensitivity of the device; the position of maxima of fluorescence is cited in the text with a consideration of spectral sensitivity.

Results and a Discussion of Them

Spectra of luminescence. From the spectra of fluorescence of mitochondria and nuclei which are presented in Figure 1 it can be seen that they are similar with the spectra of fluorescence of proteins [25, 26] and free tryptophan [26, 27]. Differences consist of a certain shift of spectra to the short-wave side. In freshly isolated preparations of mitochondria the position of the maximum varies in different animals within the limits of 350--335 nm (in two animals 350, in two 337, and in one 335 nm).

Storage of mitochondria for 24 hours at + 4° leads to a stabilization of the position of maximum at 337-345 nm. In contrast to the mitochondria the freshly isolated nuclei of all five animals possessed a stable maximum of fluorescence at 333 nm. The position of this maximum does not change during 48 hour cold storage of nuclei. The protein (tryptophan) nature of fluorescence of nuclei and mitochondria is supported by the following facts.

1. The removal initially of free nucleotides and RNA (treatment of mitochondria and nuclei with 10% perchloric acid for 18 hours at room temperature) and then of DNA (treatment with hot 5% perchloric acid for 40 min.) does not lead to any essential changes in the spectra of fluorescence. Only an insignificant shift in the maximum is observed in the long-wave range. This testifies directly to the insignificant role of all forms of nucleic acids and free nucleotides as centers of fluorescence.

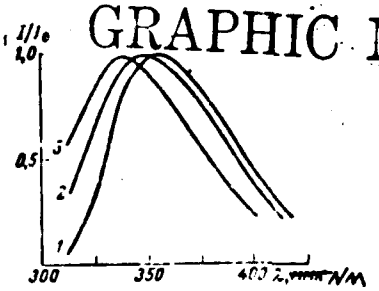

Fig. 1. Spectra of fluorescence at room temperature.
1 - aqueous solution of tryptophan (concentration of 10^{-4} M/l);
2 - mitochondria in physiological solution; 3 - nuclei in physiological solution.

The contradiction of this conclusion with the observations of Ye. M. Brumberg about the weakening in the intensity (brightness) of cell luminescence after their treatment with perchloric acid can be removed in the following manner. In place of the washing of nucleic acids as the carrier of luminescence, I. Ya. Barskiy quite correctly named as factors for this weakening the decrease in cell volume, removal of tryptophan, and disruption of supramolecular organization. To these factors one can add yet a fourth. Perchloric acid itself may cause either a chemical transformation of the tryptophan molecule

or lower its resistance to photooxidation. Actually the quantum yield of fluorescence of nuclei drops after treatment with hot perchloric acid from 2 to 1%, and after momentary irradiation in the focus of an SVD-120 A lamp by 6 more times. A decrease in quantum yield, accelerated by ultraviolet light, was observed by us also in the case of the action of perchloric acid on mitochondria, protein from fish scale, and, which is especially important, on tryptophan itself (table).

Quantum yields of fluorescence by cellular elements

(a) Объект и условия	(b) Квантовый выход	
	240 mμ	265 mμ
c Триптофан в дистиллированной воде	0,22	0,22
d Триптофан в дистиллированной воде после облучения в фокусе лампы СВД-120А в течение 1, 2, 3 и 4 мин.*	0,22; 0,16; 0,16; 0,16	0,22; 0,16; 0,16; 0,16
e Триптофан после воздействия $HClO_4$ и облучения в фокусе лампы СВД-120А в течение 1, 2, 3 и 4 мин.	0,18; 0,12; 0,09; 0,09	0,18; 0,12; 0,09; 0,09
f Триптофан в смеси с митохондриями	0,30	0,30
g Ядра, нативные в физиологическом растворе	0,02÷0,03	0,01÷0,02
h Ядра, *обработанные* $HClO_4$	0,01÷0,015	0,018÷0,015
i Ядра без *ДНК* после 1 мин. облучения СВД-120А	0,0015÷0,0025	
j Митохондрии в физиологическом растворе	0,1÷0,15	
k Митохондрии в 8 *М* мочевине	0,11÷0,17	
l Митохондрии после кипячения	0,11÷0,17	

m * Облучение производили в закрытой кювете в условиях, затрудняющих доступ кислорода воздуха, в слое полного поглощения

Key: (a) Object and conditions; (b) Quantum yield; (c) Tryptophan in distilled water; (d) Tryptophan in distilled water after irradiation in the focus of an SVD-120A lamp for 1, 2, 3, and 4 min. *; (e) Tryptophan after the influence of $HClO_4$ and irradiation in the focus of an SVD-120A lamp for 1, 2, 3, and 4 min.; (f) Tryptophan in a mixture with mitochondria; (g) Nuclei, native in physiological solution; (h) Nuclei, treated with $HClO_4$; (i) Nuclei without DNA after one min. of irradiation with the SVD-120A; (j) Mitochondria in physiological solution; (k) Mitochondria in 8 M urea; (l) Mitochondria after boiling; (m) * Irradiation was carried out in a closed cuvette under conditions, making difficult the entrance of oxygen from the air, in a layer of complete absorption.

2. Typical protein denaturing agents lead to insignificant shifts in the position of the maxima of fluorescence similar to those which are observed also in free proteins. Heating up to 100° and treatment with 8 M urea lead to a shift of the maximum to the long-wave side, a widening of the spectrum, and a certain increase in quantum yield (table).

The tryptophan nature of luminescence finds expression especially prominently in the spectra of low-temperature luminescence of

nuclei and mitochondria. Even a superficial glance at Figures 2 and 3 is sufficient to convince one that the typical tryptophan trident, which is difficult to mix up with anything else, found a clear manifestation in spectra of low-temperature luminescence. This trident together with the shoulder at 480 nm is recorded both in native nuclei and mitochondria and after removal of nucleic acids from them. Just as in the case of fluorescence at room temperature there is observed only an insignificant shift of all the maxima to the short-wave range of the spectrum. It is difficult to detect any other maxima which could be ascribed to nucleic acids in any forms whatsoever. Even the ratio of intensity of triplet and singlet maxima at low temperatures is typical for proteins [26]. This conclusion is further confirmed by experiments for studying the dependence of spectra of triplet luminescence on the wavelength of exciting light. In the range of 254-289 nm for mitochondria and in the range of 254-296 nm for nuclei the form of the phosphorescence spectrum does not depend on the wavelength of excitation (Fig. 2 and 3). This means that here, where the maximum of absorption of nucleic acids lies - at 265-270 nm, the same oscillator radiates constantly, the oscillator of tryptophan.

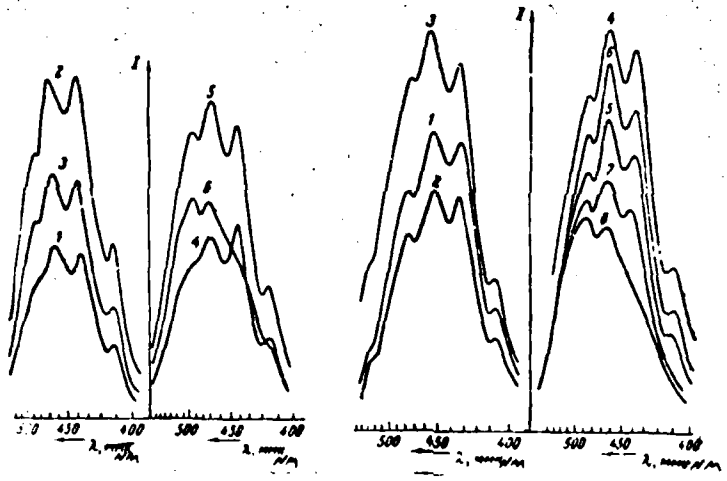

Fig. 2. Spectra of phosphorescence of nuclei in physiological solution at the temperature of liquid nitrogen depending on the wavelength of exciting light.
Wavelength of excitation, nm:
1 - 265; 2 - 270; 3 - 280;
4 - 292; 5 - 296; 6 - 302.

Fig. 3. Spectra of phosphorescence of mitochondria in physiological solution at the temperature of liquid nitrogen depending on the wavelength of exciting light.
Wavelength of excitation, nm:
1 - 265; 2 - 270; 3 - 280;
4 - 289; 5 - 292; 6 - 296;
7 - 302; 8 - 313.

The picture is somewhat different in the range of excitation of 292-313 nm. In this range with the invariability of the position of maxima of triplet luminescence there is observed a redistribution of their intensities; maxima are heightened sharply at 465 and 480 nm due to the weakening of maxima at 405 and 431 nm. Apparently such a redistribution in probabilities of transitions with an invariability of position in the energy scale may be attributed to several factors: the existence of two oscillators responsible for the phosphorescence of tryptophan, the existence of tryptophan in two forms, and deformations in the system of electron fluctuating sublevels of the tryptophan molecule due to surplus energy of absorption. A choice between these three possibilities is difficult at present, though we are inclined more to the thought of two forms of tryptophan. In any case the influence of the site of absorption on the ratio of intensities of maxima of triplet luminescence is inherent to the very molecule of tryptophan and not to other centers of luminescence which enter into play as a measure of movement along the scale of wavelengths, since the redistribution of intensities of maxima is also observed for tryptophan itself under certain conditions (the results are being prepared for publication).

Thus, based on the spectra of luminescence it is hardly possible to connect the fate of quantum which is generated in the act of photoluminescence with centers other than the tryptophan of proteins. This conclusion finds a well-defined confirmation in the spectra of fluorescence excitation, which simultaneously tell us which molecules absorb that light energy which is released in the form of luminescence (it can be imagined, for example, that quanta of light which are scintillated by tryptophan were initially absorbed by nucleic acids and then relayed to tryptophan in the act of migration of energy).

Spectra of excitation of luminescence. Figure 4 shows the spectra of excitation of fluorescence of mitochondria and nuclei in the range of 217-302 nm. In both spectra both maxima of absorption of proteins are clearly manifested: at 280 and at 227-230 nm, separated by the minimum at 248 nm. In the range of wavelengths greater than 300 nm the excitation of luminescence is absent. However attention is merited by the fact of the insignificance of the shielding effect by nucleic acids on the tryptophan in the nuclei. The quantum yield of fluorescence for nuclei for 265 nm all told is 50% lower than the quantum yield for 280 nm (table). This circumstance does not exclude the possibility of partial migration of energy from nucleic acids to the proteins.

Polarization spectra of fluorescence. Polarization spectra of fluorescence of tryptophan and proteins were investigated in the works by Weber [28] and Konev and Katibnikov [29].

Polarization spectra, in reflecting the spatial anisotropy of the mutual position of oscillators of absorption and emission, are very sensitive to an admixture of "extraneous" centers of luminescence. Nevertheless Figure 5 clearly demonstrates the typically protein (tryptophan) nature of the polarization spectrum both of the fraction of mitochondria and the fraction of nuclei.

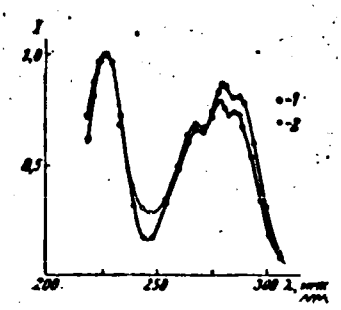

Fig. 4. Spectra of action of fluorescence of mitochondria (1) and nuclei (2) in physiological solution at room temperature.

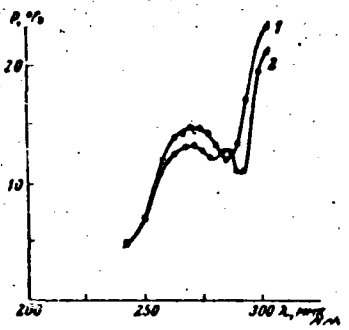

Fig. 5. Polarization spectra of fluorescence (based on absorption) of mitochondria (1) and nuclei (2) in physiological solution at room temperature.

In the spectra a maximum is manifested at 265-270 nm, a dip in the range 280-285 nm, and a considerable increase in the long-wave range (296-300 nm). The analogous form of the long-wave section of the spectrum of tryptophan in glycerin solutions in a comparison with the spectrum of absorption in this range testifies, apparently, to the correctness of the proposal by Weber that beyond the band of absorption of tryptophan at 280 nm there are hidden two differently oriented oscillators of absorption /28/. At the same time it is seen from the polarization spectra that the degree of polarization of luminescence of mitochondria, decreasing strongly along the entire spectrum in comparison with free tryptophan, is practically equal to the degree of polarization of protein solutions. For 265 nm we obtained values for the degree of polarization of luminescence of human serum albumin of 15.0%, and of protein extract from mitochondria - 15-16%. Therefore, for at least the overwhelming majority of proteins, under the conditions of the structural organization of mitochondria the migration of energy between radicals of tryptophan which belongs to neighboring macromolecules of protein does not take place.

Thus without exception all the spectral characteristics speak for the protein (tryptophan) nature of ultraviolet luminescence of the main organoids of hepatic cells - mitochondria and nuclei. Apparently the fraction of microsomes which is being investigated at present has an analogous nature. Here we are naturally aware that the results do not exclude the possibility of the existence of other carriers of luminescence (for example, nucleic acids) in

other objects and in the same objects under different conditions. Even in the case investigated by us the weak luminescence of many substances apparently can be masked by the more intensive protein luminescence. Without a doubt other centers of luminescence appear, for example, during transition to longer-wave excitation as this was observed by us during excitation of frozen nuclei with light at 365 nm, when luminescence appeared with a maximum at 490 nm, which most likely belongs to pyridine nucleotides.

Conclusions

1. A study is made of the spectra of luminescence at room and low temperatures with integral and monochromatic excitation. Also studied were the spectra of excitation of luminescence and polarization spectra of luminescence (based on absorption) of isolated fractions of mitochondria and nuclei from the hepatic cells of white rats.

2. The spectra show that the luminescence of mitochondria and nuclei of these cells, which was excited in the spectral range of 250-300 nm , is caused exclusively by proteins (tryptophan).

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